CYGNUS TECHNOLOGIES
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Product Manual: D1555W

CHO AccuRes™ DNA Quantification Kit in Wells

Contents

Intended Use

This kit is intended for use in determining the presence of host cell DNA contamination in products manufactured by recombinant expression in CHO cell lines. This kit contains reagents for DNA extraction as well as calibrated DNA concentrate, primers and probe mix, and 2x PCR Master Mix for DNA amplification.

The kit is for **Research, Development and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary & Explanation

Expression of therapeutic proteins in CHO cells is a cost-effective method for production of commercial quantities of a drug substance. However, the manufacturing and purification process of these products leaves the potential for residual DNA contamination from the host cells. Due to the theoretical potential for the transfer of oncogenes from the host cell, both the WHO and FDA have set the allowable limit as 10ng/dose.

This kit is designed to measure CHO host cell residual DNA for the purpose of process development, in-process monitoring, and QC lot release testing. Quantitative PCR (qPCR) based assays have been employed by many biopharmaceutical manufacturers over the years. However, in many cases proteins and buffer components can interfere with DNA amplification, resulting in either over or under estimation of the true DNA concentration. This kit uses a proprietary DNA extraction procedure to isolate the residual DNA from complex matrices and perform the measurements in an environment free from contaminating proteins, salts and detergents. The removal of contaminating product protein and other excipients ensures accurate measurements of residual host cell DNA and allows for timely and scientifically sound process decisions.

Principle of Procedure

This convenient, easy-to-use kit is compatible with your existing qPCR instruments. In addition to all materials required for Cygnus' proprietary DNA extraction procedure, which removes PCR interfering components from samples in a high-throughput 96 deep well plate, the kit also includes DNA concentrate for preparation of standards, a cell-line specific AccuRes™ CHO PCR Primers and Probe mix, 10X, and AccuRes™ PCR Master Mix, 2X. Upon completion of extraction of residual DNA, the sample, standards, and controls are transferred to a qPCR plate containing AccuRes™ Primers and Probe Mix with PCR Master Mix. After sealing the plate with the supplied optical seal, the qPCR plate is subjected to 40 amplification cycles. A standard curve is constructed from the point at which each standard crosses a pre-established threshold. The samples and controls are measured against the standard curve to determine the concentration of DNA. Using this method, residual DNA can be measured to a limit of detection (LOD) of 0.6 femtogram per microliter levels.

Reagents & Materials Provided

Component	Product #
DNA Extraction:	
Proteinase K, 1 x 150µL	D101
DNA Extraction Buffer, 1 x 30mL	D105*
DNA Precipitation Buffer, 1 x 55mL	D106*
DNA Wash Buffer, 1 x 170mL	D104
DNA Sample Diluent, 1 x 30mL	D006*
Deep Well Extraction Plate with Sealing Mat, 1 x 96 wells	D102*
DNA Amplification:	
CHO DNA Concentrate, 100ng/10µL x 120µL	D556*
AccuRes™ PCR Master Mix, 2X, 2 x 750µL	D1005
AccuRes™ CHO PCR Primers and Probe Mix, 10X, 1 x 350µL	D1557
AccuRes™ Deionized Water, 2 x 500µL	D1006
PCR Assay Plate with Optical Seal, 1 x 96 wells	D004*

*Component can be purchased separately.

Storage & Stability

The AccuRes™ CHO PCR Primers and Probe Mix, 10X, and AccuRes™ PCR Master Mix, 2X, should be stored at -20°C for long-term storage. After thawing, The AccuRes™ CHO PCR Primers and Probe Mix, 10X, and AccuRes™ PCR Master Mix, 2x can be stored at 2°C to 8°C for stability until the expiration date printed on the kit label. If you are storing DNA samples for testing/retesting, we recommend short-term storage at 2-8˚C.

Materials & Equipment Required but Not Provided

- DNA TE Buffer, Cat# D001 (Tris/EDTA used for Proteinase K dilution and final pellet reconstitution)
- Uracil-DNA Glycosylase (NEB M0280L) for amplicon contamination control (if desired)
- Pipettors – 5µL – 1200µL
- Bench top microfuge capable of spinning a microplate at 3,200 x g.
- Microtiter plate shaker with 1,000 rpm capability. (Recommended: THERMO Titer Plate Shaker, Model: 4625)
- Dry heat block for use with microplates
- Vortex
- Plate sealer/roller
- Spray bottle
- Absorbent wipes
- Real-time PCR instrument capable of detecting FAM signal

Precautions

- For Research, Development or Manufacturing use only.
- This kit should only be used by qualified technicians.

Preparation of Reagents

Bring all extraction reagents to room temperature prior to starting the extraction procedure. When the DNA extraction is completed and you are ready to perform the amplification step, the standards and PCR plate can be prepared at room temperature.

Preparation of Standards

Prepare the Standard Curve by making 10-fold dilutions of the DNA Concentrate according to the table. Discard tubes 1-3.

Tube #	CHO DNA	Sample Diluent	Final Concentration
1	Stock	N/A	100 ng/10 µL
2	100 µL of Tube 1	900 µL	10 ng/10 µL
3	100 µL of Tube 2	900 µL	1 ng/10 µL
4	100 µL of Tube 3	900 µL	100 pg/10 µL
5	100 µL of Tube 4	900 µL	10 pg/10 µL
6	100 µL of Tube 5	900 µL	1 pg/10 µL
7	100 µL of Tube 6	900 µL	0.1 pg/10 µL
8	100 µL of Tube 7	900 µL	0.01 pg/10 µL
9	0 µL	900 µL	0 pg/10 µL

Preparation of Samples

If the sample contains more than 10 mg/mL of protein, we recommend diluting the sample in sample diluent (Cat # D006); see procedural note #1 below.

Amplification Reagent Preparation

Prepare the amplification reagent mixture using the below volumes per well for the qPCR assay, adding ~10% overage to accommodate for pipetting error.

Reagent	Volume/well
AccuRes™ PCR Master Mix (2X)	**15 µL**
AccuRes™ Primers and Probe Mix (10X)	**3 µL**
AccuRes™ Deionized Water	**2 µL**

Procedural Notes

1. Protein in the sample is a known interference factor in DNA quantification methods. Use of the proprietary Cygnus DNA Sample Diluent (Cat# D006) will generally allow for acceptable DNA recovery in up to 10mg/mL of protein. Samples with higher protein concentrations can be used but must be qualified in the assay.
2. Due to the extreme sensitivity of this assay, it is important to keep the working area clean to avoid contamination by DNA in the environment. Thoroughly clean pipettes and the immediate working area prior to initiating the procedure. Remove anything from the area that is not required for the procedure. Avoid leaning over the extraction plate as much as possible; organize solutions and tips such that passing over the plate is minimized.
3. Proteinase K digestion should be carried out at 60°C for most therapeutic proteins. Monoclonal antibodies generally perform well at 60°C. However, each laboratory may need to determine the optimum temperature for non-IgG drug products. Do not exceed 60°C as this may cause the protein to precipitate from solution.

4. Always make sure the centrifuge is balanced to ensure proper assay performance. An improperly balanced centrifuge can result in loose pellets, which can adversely affect recovery and assay precision.
5. We recommend using a plate sealer/roller to ensure a secure fit of the Sealing Mat onto the 96 deep well plate.
6. While it is possible to seal off wells for use of a partial plate, Cygnus recommends using a new plate for each DNA extraction to prevent contamination. Please visit our website to purchase additional Deep Well Extraction plates, Catalog # D102.
7. Complete removal of residual liquid from the wells after each spin is essential for proper assay performance. Ensure that the wells are free of all visible liquid immediately following the wash steps, either through inverting/tapping or pipette removal.
8. Cygnus Technologies has determined that various brands of paper towels have different levels of lint and dust. Even towels and wipes that claim to be lint-free can affect results. To prevent lint and dust from entering your sample, we suggest gently misting the towels with either TE (10mM Tris, 1 mM EDTA) or distilled water with a standard spray bottle prior to tapping out the tubes or plate. Alternatively, a pipette may be used to remove the liquid after the precipitation step.
9. The CleanAmp® dNTP mix contained in AccuRes™ PCR Master Mix (2X) includes CleanAmp®dUTP, which provides the opportunity to treat the reaction with UNG/UDG (not included) to eliminate contamination with other amplicons.
10. The DNA probe contained in the AccuRes™ Primers and Probe Mix is conjugated to a fluorophore (FAM) and Black Hole Quencher™ (BHQ™). During PCR extension, the probe is cleaved from the quencher, allowing for the release of quantifiable fluorescent signal.
11. ROX reference dye is included in the AccuRes™ PCR Master Mix to ensure normalization across sample wells during PCR amplification.
12. We recommend testing each sample and control in triplicate to ensure detection and quantification is as sensitive and robust as possible.

Limitations

The AccuRes™ DNA Quantification kit is available in two formats, depending on the preferred vessel for the DNA extraction procedure. This kit is designed to accommodate DNA extraction in a 96 deep well microplate. If you do not have the ability to spin deep well plates at 3,200 x g and/or would prefer to perform the extraction in 2 mL microfuge tubes, please order AccuRes™ CHO DNA Quantification Kit in Tubes (Cat# D1555T).

Calculation of Results

The C_T values of the standards are used to construct a standard curve with values reported by the instrument in pg/10µL host cell residual DNA. The concentration of host cell residual DNA can be mathematically transformed for reporting residual DNA in ng/mL, ng/mg of drug product or in ng/dose.

DNA Extraction Protocol

1. **Proteinase K must be diluted fresh for each assay run. Prepare only the amount of 1:10 diluted Proteinase K required for that run. For example, if the assay requires 50 wells, add 75µL of Proteinase K to 675µL of DNA TE Buffer, (Cat # D001) or other qualified TE buffer.**
2. **Dilute all test samples to DNA concentrations within the analytical range of the curve and to < 10mg/mL total protein using DNA Sample Diluent (Cat# D006). All samples should be diluted at least 1:2.**
3. **Transfer 250 µL of each test sample, standard, and control to the deep well plate (columns 4-12) and perform all required spiking and diluting. If samples are being diluted in the plate, ensure the final volume is 250 µL.**
4. **Add 12.5µL of diluted Proteinase K to each test sample well. Mix by gently pipetting or by shaking (30 sec) on the plate shaker.**
5. **Seal the plate with the sealing mat and incubate the standards, samples, and controls at 60°C for 30 minutes in a dry heat block with a microplate adapter.**
6. **Centrifuge the plate for 1 minute at 3,200 x g to recover any condensation on the sealing mat.**
7. **Add 250 µL of Extraction Buffer to standards, controls, and samples and mix thoroughly. Reseal the plate and shake (~800 rpm) for 5 minutes.**
8. **Add 500 µL of Precipitation Buffer to each well and mix thoroughly. Reseal the plate and shake (~1,000 rpm) for 15 minutes.**
9. **Centrifuge the plate at 3,200 x g for 20 minutes.**
10. **Decant supernatant and remove additional liquid by tapping the plate upside down on lightly misted lint-free wipes until free of all visible liquid. Alternatively, supernatant can be removed using a pipette. Inspect each well carefully to ensure no visible liquid remains.**
11. **Add 0.8 mL of DNA Wash Buffer to each well and shake (~800 rpm) for 5 minutes.**
12. **Centrifuge at 3,200 x g for 5 minutes.**
13. **Decant supernatant and remove additional liquid by tapping the plate upside down on lightly misted lint-free wipes until free of visible liquid. Alternatively, liquid can be removed with a pipette tip, followed by a brief incubation and visual inspection to ensure the pellets are completely dry.**
14. **Repeat steps 11-13.**
15. **Re-suspend the pellets in 125 µL of DNA TE Buffer (Cat# D001) pre-warmed to 50°C (to aid in dissolving the pellet). Incubate on the shaker (~800 rpm) at room temperature for 5-10 minutes. Mix gently with a pipette to further aid resuspension if desired.**
16. **The CHO DNA is now ready for downstream qPCR amplification.**

DNA Amplification Protocol

1. **The amplification reagent is prepared by combining Cat# D1557 AccuRes™ CHO Primers and Probe Mix, 10X (3 µL/well), Cat# D1005 AccuRes™ PCR Master Mix, 2X (15 µL/well), and AccuRes™ Deionized Water (2µL/well) as described in the 'Amplification Reagent Preparation' section on page 5.**
2. **Prepare 20µL of the amplification reagent for each well plus an additional 10% excess. For example, 96 wells + 10% = enough amplification reagent for 105 wells.**
3. **Transfer 20µL of amplification reagent to each well of the qPCR assay plate (Cat# D004).**
4. **Transfer 10µL of each standard, test samples and controls to the qPCR assay plate. We recommend testing each sample in triplicate.**
5. **Apply the optical seal over the wells.**
6. **Gently tap the side of the plate to remove all bubbles from the bottom of the wells.**
7. **Place the assay plate into the qPCR instrument.**
8. **Suggested amplification parameters:**

   Step 1: 37°C 2 minutes (optional)

   Step 2: 95°C for 10 minutes.

   Step 3: 95°C for 15 seconds followed by 60°C for 1 minute (x 40 cycles).

Performance Characteristics

Cygnus Technologies has qualified this assay by conventional criteria as indicated below. A more detailed copy of this "Qualification Summary" report can be obtained by request. This qualification is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform a spike and recovery study for each sample type to be tested in the assay. Each laboratory technician should also demonstrate competency in the assay by performing a similar precision study to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Department or at our web site.

qPCR Amplicon

The AccuRes™ CHO PCR Primers and Probe Mix provided in this kit will amplify a multi-copy Alu element specific to CHO DNA, producing an amplicon of 100 base pairs.

Accuracy and Precision

CHO DNA samples were prepared in a 1 mg/mL human IgG sample matrix containing DNA Sample Diluent (Cat# D006) at various concentrations spanning the Standard Curve. Three preparations were made for each sample and duplicate wells were collected for each preparation resulting in 12 individual results per concentration. The assays were performed over 2 days.

Concentration	% Nominal	Intra-assay CV	Inter-assay CV
50pg/10µL	90%	6.0%	5.2%
5pg/10µL	96%	3.2%	2.9%
0.5pg/10µL	99%	3.3%	1.8%
0.05pg/10µL	91%	8.7%	8.5%

Ordering Information/ Customer Service

To place an order or to obtain additional product information, contact:

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Need a Custom DNA Assay developed? - Contact Cygnus Experts

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